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The Effects of Prolonged Treatment with Acriflavine on the Killifish, *Fundulus heteroclitus* (Linnaeus)

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THE experiments reported below were undertaken to determine whether prolonged treatment with acriflavine, used in an attempt to control an infectious epidemic similar in its symptoms to that described by Wells & Zobell (1934) for *Fundulus parvipennis* but not curable by the heat treatment recommended by these authors, was responsible for the development of cirrhosis of the liver due to the accumulation of large amounts of a ceroid-like material.

The bacteriocidal and trypanocidal acriflavine dyes (acriflavine, trypaflavine, euflavine) are commonly used in the treatment of protozoan infections of fish. Schäperclaus (1941), for example, recommends a bath in solutions containing 1 gm in 50 to 100 liters of water for ten hours, but the dye may be allowed to remain in the water until the color "wears off" (Nigrelli, personal communication). The latter procedure had been used in the treatment of infected fish which, at autopsy, showed the liver condition mentioned above. It was thought that the acriflavine might have a direct action or, alternatively, that it might act indirectly through the elimination or partial elimination of the normal intestinal bacterial flora which, as is well known in higher vertebrates, may play an important role in supplementing the natural source of vitamins derived from the food. The experiment was designed to test both these hypotheses.

My best thanks are due to the staff of the Bingham Oceanographic Laboratory, especially to my former assistants, Miss Harriet A. Chambers and Mrs. Shirley M. Conover, and to Mr. A. Charles Laws, all of whom participated in the preparation of the tissues, and to Mrs. Joyce Lownsbery who assisted with the statistical calculations. I am also indebted to Dr. Ross F. Nigrelli, Dr. Donald F. Poulson, and Dr. Daniel Merriman for many valuable suggestions.

Materials and Methods

Four tanks each containing 52 liters of sea water were arranged as follows: Tanks 9 and 10 had plain sea water, Tanks 11 and 12 each received in addition 10.4 cc of 1% neutral acriflavine so that the approximate strength of the dye was 2 gm per 1,000 liters. On Oct. 26, 1950, eight two-year-old specimens of *Fundulus heteroclitus* were placed in each tank. All were supposedly males but, at this time of year, the sexes are difficult to separate and at autopsy one fish in Tank 9 was discovered to be a female. Each fish was marked with colored beads sewn on with tantalum wire, and its weight and length were recorded. The fish were in complete sexual regression with at most a mere trace of nuptial coloration. During the course of the experiment one fish died in Tank 11, from unknown causes. The female in Tank 9 has been excluded from the analysis.

The color of the water in the tanks containing acriflavine faded slowly with time and 10 cc of 1% stock solution were added on the following dates: November 14, January 8, February 11 and March 4.

The fish were fed daily, with rare exceptions. All tanks received a regular diet of cooked liverpablum (Gordon, 1943). Tanks 10 and 12, one without and one with acriflavine, received nothing but this diet during the whole course of the experiment. In accordance with the plan outlined above, Tanks 9 and 11 received a dietary supplement intended to provide an additional source of vitamins. This supplement was offered about thrice weekly and consisted at first of chopped raw beef liver but, beginning on February 11, this was replaced with frozen, raw daphnia in generous amounts. Also, after February 11 a trace of iodine was added to the liverpablum, since results of previous experiments, which became available at this time, showed that

Table 1. Summary of the Experimental Results

	No acriflavine		Acriflavine	
	Daphnia	No daphnia	Daphnia	No daphnia
Tank	9	10	11	12
No. of fish	7	8	7	8
Weight % incr.	40.0±10.7	44.2±13.3	25.1±4.8	28.0±11.9
Length % incr.	14.4±6.5	10.3±2.4	6.9±1.4	9.3±4.9
Testis GSI[1]	3.9±0.9	4.2±2.4	3.5±1.2	1.4±0.4
Nuptial colors	Strong	Strong	Strong	Slight
Liver HSI[2]	3.5±0.6	3.8±0.7	3.4±0.5	3.9±0.7
Liver color	Pinkish-brown	Pinkish, reddish, or grayish-brown	Pinkish, reddish, or grayish-brown	Ochraceous
Liver glycogen	Abundant	Abundant	Abundant	Abundant
Liver fat	Abundant	Abundant	Abundant	Abundant
Liver ceroid	Trace	Trace	Trace	Trace
Thyroid epith. ht.[3]	5.4±0.7	5.0±0.6	4.6±0.6	5.2±0.5
Thyroid active colloid[4]	4 fish	3 fish	2 fish	1 fish

[1]GSI = gonosomatic index.
[2]HSI = hepatosomatic index.
[3]Thyroid epith. ht., standard deviation calculated for the differences between the means for each fish.
[4]Thyroid active colloid: predominently blue or particolored with Azan.

fish fed exclusively on this diet tended to develop a low to moderate grade of goiter.

On April 2-3, 159-160 days after the start of the experiment, the fish were killed and subjected to autopsy. Weight, length and nuptial coloration were again recorded. The liver was weighed and its size expressed in terms of the hepatosomatic index. Liver color was noted against the Ridgway color chart, and slices were fixed in alcohol-formalin (for glycogen) and calcium formol (for fat). Glycogen was estimated subjectively on sections stained by the periodic-Schiff method, with malt diastase controls. Fat was similarly estimated on frozen sections stained with scarlet red and haematoxylin, control sections being treated with scarlet red dissolved in 100% alcohol which removes most if not all of the soluble fat in one hour. Ceroid was stained red by the periodic-Schiff method on the glycogen controls and remained as undissolved material stained by scarlet red on the fat controls.

The development of nuptial coloration is a visible indication of the maturation of the male gonad. In addition, the testes were weighed and the relative size expressed in terms of the gonosomatic index. The hypobranchial region was fixed in Bouin's fluid and the thyroid was studied on serial sections, alternate slides being stained with haematoxylin-eosin and with Azan. The height of a single follicle cell was measured from each follicle on every twentieth section and the average cell height calculated from 100 cells for each fish.

Results

The overall results are summarized in Table I.

Weight.—Fish in the two acriflavine tanks showed a retardation of growth as measured by percentage of weight increase. The dietary supplement had no effect on untreated fish and conferred no beneficial effect in the presence of acriflavine.

Length.—Effects on growth in length are more difficult to interpret. The best growth was obtained in Tank 9 (daphnia, no acriflavine) but there is no significant difference between Tanks 9 and 10, although growth in the latter was somewhat less. Therefore, in the absence of acriflavine the dietary supplement had little or no effect. Similarly when Tanks 11 and 12 are compared there is no significant difference. However, the fish in Tank 11 (daphnia and acriflavine) showed a very marked retardation of growth in length, which is significantly different from that in either Tanks 9 or 10 (no acriflavine). Contrary to expectation, and in disagreement with the results based on weight increase, the length increase in Tank 12 is not statistically less than that in Tanks 9 and 10. The less striking retardation of growth in Tank 12 may be correlated with the inhibition of sexual development discussed below.

The values of P in the comparison of length increase are given in Table 2. Analysis of variance supports the hypothesis that the four tanks are not parts of a homogenous population ($F = 5.40$, $df = 3$ and 26) but, in view of the

small numbers, a more detailed study was not attempted. It is felt that a larger series would show that growth in length, like growth in weight, is retarded by the presence of acriflavine.

Sexual development—It was evident by inspection that the fish in Tank 12 were in a state of partial sexual regression, as manifested by the weak development of nuptial coloration. This was fully confirmed by the low values of the gonosmatic index. The small differences between Tanks 9, 10 and 11 are not statistically significant. These results indicate that sexual retardation due to treatment with acriflavine may be abolished by a diet enriched with raw daphnia. The effects on the testes are therefore indirect or moderated by a more efficient metabolism of the dye.

Table 2. Values of P in Comparisons of Growth in Length

	Tank 9	Tank 10	Tank 11
Tank 10	0.1-0.2	—	—
Tank 11	0.01	0.001-0.01	—
Tank 12	0.1	0.6-0.7	0.2-0.3

Liver.—It had been anticipated at the start of the experiment that the liver would show marked differences correlated with the acriflavine treatment, hence the careful study of fat and glycogen. The results do not confirm this expectation. In all groups the hepatosomatic indices were in the normal range for spring males. There is a slight tendency for the fish in Tanks 10 and 12, which did not receive a dietary supplement, to have larger livers, but the differences are not statistically significant. Similarly the study of sections revealed no appreciable differences in fat or glycogen content. All livers were heavily laden with reserves, showing the fish to be in a well-nourished condition. It is probable that if the experiment had been continued through the advancement of the breeding season the fish with well-developed gonads would have developed a depletion of liver reserves similar to that which is known to occur in the wild population (Chambers, 1951; Pickford, 1952). No fish showed cirrhosis of the liver and the small amounts of ceroid which were present in all cases are such as occur normally in wild fish.

The data regarding liver weight, and glycogen and fat content, do not appear to tell the whole story. The livers of fish from Tank 12 were all more or less strongly ochraceous in color and after fixation a yellow, non-fluorescent, water soluble coloring matter dissolved out into the fixing fluid. The livers of fish in the three remaining tanks showed the normal range of pinkish or reddish-brown, with an occasional grayish-brown, and did not yield this bright yellow solution after fixation. It appears that in the absence of a suitable dietary supplement the livers are unable to metabolize the acriflavine in a normal manner.

Thyroid.—In contrast to earlier experiments in which no iodine had been added to the liver-pablum mixture, no fish developed goiter. The condition of the thyroids is essentially the same in all tanks. The slightly lower epithelial height noted in the fishes of Tank 11 is just barely statistically significant, at the 5% level, when this group is compared either with Tank 9 or with Tank 12, but it is not significantly different from Tank 10. Analysis of variance does not support the hypothesis that the Tank 11 group is significantly different from the population as a whole ($F = 2.09$, $df = 3$ and 26). No correlation was observed between the average epithelial height and the condition of the colloid as seen after staining with Azan. In most cases the thyroid follicles were predominantly inactive, with homogenous, red-staining colloid. In each tank some individuals showed a more active condition, with predominantly blue-staining, granular, or parti-colored colloid. The incidence of active colloid was certainly higher in those groups whose growth was not retarded by acriflavine.

Discussion

In a study of the antibacterial action of acriflavine, McIlwain (1942) has shown that inhibition of growth caused by this dye may be reversed by nucleic acid and to a lesser extent by degradation products of nucleic acid. Acriflavine forms complex salts with nucleic acid, lacking the fluoresence of the free substance. A second type of reversal could be obtained with marmite and with phenylalanine, the effect being augmented by riboflavin and other hydrogen carriers such as methylene blue. McIlwain concludes that the inhibited system lacks both substrate and normal H-transport mechanisms and that acriflavine probably combines with enzyme systems involving nucleotides. As McIlwain points out, the inhibitory action of acriflavine on cell respiration is well known; the work of Quastel & Wheatley (1931) on bacteria is cited, also the work of Scheff & Hassko (1936) on trypanosomes. The latter authors conclude that the effect is on a cyanide-sensitive hydrogen carrier. More recently Ephrussi and co-workers (Ephrussi, 1950, and references

therein) have shown that acriflavine induces an inheritable cytoplasmic mutation in yeast. The mutants lack cytochrome oxidase and succinic dehydrogenase, and are unable to utilize glycogen except by anaerobic metabolism.

A biochemical study was beyond the scope of the present investigation, although an examination of the respiratory enzyme systems of the liver in acriflavine-treated fish would doubtless be most illuminating. The inhibitory action on the growth of the fish may be a direct effect, since it cannot be offset by a dietary supplement of raw daphnia. The pronounced retardation of the maturation of the testes is in a different category since it may be prevented by a suitable enrichment of the diet. It is suspected that this may be due to a vitamin deficiency but it is also possible that raw daphnia provides factors similar to those noted by McIlwain in marmite which restore the growth of acriflavine-inhibited bacteria.

The retardation of the gonads supports a popular belief, to which I can find no scientific reference, that treatment with acriflavine causes temporary sterility in fish[1]. However, it is known from the work of Hertwig (1924) and Dalcq (1931) that spermatozoa may be inactivated by acriflavine, although the mechanism of this inactivation has not been elucidated.

In respect to the development of hepatic cirrhosis, towards which the investigation was primarily directed, the results are entirely negative. This disease is not caused by the presence of acriflavine in the water, nor by the prolonged use of a cooked liver-pablum diet without supplementary sources of vitamin. The problem of the development of ceroid in the liver of *Fundulus* is still under investigation.

Summary

1. Prolonged treatment with acriflavine retarded the growth of *Fundulus heteroclitus* on a standard cooked liver-pablum diet. This effect was not offset by a dietary supplement of raw, frozen daphnia.
2. Acriflavine caused a marked retardation of the testes, prevented by the addition of raw daphnia to the standard diet.
3. Acriflavine had no effect on liver weight, nor on the glycogen and fat reserves of the liver. It did not induce hepatic cirrhosis resulting from the accumulation of ceroid in the liver. However, in the absence of raw daphnia, a liver disfunction was indicated by the accumulation of a yellow, non-fluorescent, water soluble coloring matter.
4. Acriflavine had little or no effect on the function of the thyroid gland as reflected in average follicle cell height and colloid condition.

[1]In the popular aquarium literature, Earle E. Patterson (Aquarium Journal [San Francisco], Vol. 21, p. 36, 1950) has reported a loss in reproductive capacity in fishes treated with acriflavine.

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